Gut microbiota and nuclear receptors in bile acid and lipid metabolism

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CHAPTER 3

Liver receptor homolog-1 is critical for adequate upregulation of Cyp7a1 gene transcription and bile salt synthesis during bile salt sequestration

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Hepatology, 2011 Jun;53(6):2075-85.

ABSTRACT

Liver receptor homolog-1 (LRH-1) is a nuclear receptor that controls a variety of metabolic pathways. In cultured cells, LRH-1 induces the expression of CYP7A1 and CYP8B1, key enzymes in bile salt synthesis. However, hepatic *Cyp7a1* mRNA levels were not reduced upon hepatocyte-specific *Lrh-1* deletion in mice. The reason for this apparent paradox has remained elusive. We describe a novel conditional whole-body *Lrh-1* knockdown (LRH-1-KD) mouse model to evaluate the dependency of bile salt synthesis and composition on LRH-1. Surprisingly, *Cyp7a1* expression was increased rather than decreased under chow-fed conditions in LRH-1-KD mice. This coincided with a significant reduction in expression of intestinal *Fgf15*, a suppressor of *Cyp7a1* expression, and a 58% increase in bile salt synthesis. However, when fecal bile salt loss was stimulated by feeding the bile salt sequestrant colesevelam, *Cyp7a1* expression was upregulated in wildtype mice but not in LRH-1-KD mice (+593% in wildtype versus +9% in LRH-1-KD). This translated into an increase in bile salt synthesis of +272% in wildtype versus +21% in LRH-1-KD mice. Conclusion: Our data provide mechanistic insight into a missing link in the maintenance of bile salt homeostasis during enhanced fecal loss and support the view that LRH-1 controls *Cyp7a1* expression from two distinct sites, i.e., liver and ileum, in the enterohepatic circulation.

INTRODUCTION

Bile salts are synthesized from cholesterol exclusively in the liver by a complex multienzyme process. Crucial steps in the synthesis pathway comprise the addition of one or two hydroxyl groups to the sterol nucleus and the oxidative cleavage of the side chain of cholesterol, resulting in a highly amphipathic class of bile salt molecules. Bile salts are potent surfactants that solubilize phosphatidylcholine and cholesterol in bile and promote lipid absorption in the small intestine. Next to being the primary driving force for hepatic bile formation, the role in intestinal lipid digestion has long been thought to be the most important function of bile salts (1). The landmark discovery of bile salts as endogenous ligands for the nuclear hormone receptor farnesoid X-receptor (FXR) and, more recently, for the G-protein-coupled receptor TGR5 has completely transformed the field of bile salt research. In addition to mediating the feedback control of bile salt synthesis, FXR influences many pathways involved in lipid metabolism and has recently also been implicated in control glucose metabolism (2). TGR5 seems particularly important in regulating energy metabolism (3).

Accordingly, it is essential to fully understand the factors that regulate synthesis of the various types of bile salts. Liver receptor homolog-1 (LRH-1) has been implicated herein, but its exact role has remained elusive so far. LRH-1 belongs to the NR5A family of nuclear receptors together with steroidogenic factor-1 and the *Drosophila melanogaster* ortholog Fushi tarazu factor-1 (4-6). In contrast to most other nuclear receptors, members of the NR5A subfamily bind DNA as monomers (7, 8) LRH-1 is essential for embryogenesis, as targeted gene disruption results in early embryonic lethality.9 In the adult mouse, LRH-1 is expressed predominantly in the ovaries, the exocrine pancreas, and the organs that constitute the enterohepatic axis, i.e., liver and small intestine (9-11). In the small intestine, LRH-1 has been shown to stimulate cell proliferation in intestinal crypts (12) and to regulate extra-adrenal glucocorticoid synthesis (13) that protects against inflammatory bowel disease (14). In line with these antiinflammatory effects, hepatic LRH-1 acts as a potent suppressor of the acute phase response (15, 16). Functional LRH-1 binding sites have been found within the promoter regions of several genes implicated in lipid metabolism and transport such as *Abcg5/Abcg8*, *APOA1*, and *SR-B1* (17-19).

LRH-1 has been proposed to function as an important transcription factor in control of bile salt synthesis. The first and rate-controlling step in the classic pathway of bile acid synthesis is catalyzed by the enzyme cholesterol 7α-hydroxylase (CYP7A1)(20). Subsequently 7α-hydroxycholesterol is converted into cholic acid by 12α-hydroxylase (CYP8B1), which determines the ratio in which the primary bile salt species cholate (3α,7α,12α-trihydroxy-5β-cholate) over chenodeoxycholate (3α,7α-dihydroxy-5β-cholate) are being produced (21).

Hepatic bile salt synthesis is tightly regulated by complex feedback mechanisms involving the consecutive and/or simultaneous actions of a number of hepatic nuclear receptors and transcription factors such as LXR, SREBPs, and HNF4 (3, 22-25). In addition, LRH-1 binding sites have been identified in the proximal promoter parts of CYP7A1 and CYP8B1 (8, 26). Data from cell studies showed that LRH-1 is able to induce the expression of CYP7A1 (8, 22, 23) and CYP8B1 (26). Therefore, LRH-1 has been proposed to function in feedback regulation of CYP7A1 expression as part of the FXR-SHP-LRH-1 cascade, in which bile acids can inhibit their own synthesis. In this cascade bile salt-activated hepatic FXR induces the expression of small heterodimer partner (SHP) that functions as a potent repressor of hepatic LRH-1 activity (27) which then results in less activation of CYP7A1 by LRH-1. In addition, upon activation of intestinal FXR, the endocrine growth factor FGF15 is produced and transported to the liver, where it binds its receptor FGFR4 and represses CYP7A1 expression in the liver (28, 29). Thus, bile salt synthesis is under negative feedback control from at least two distinct sites in the enterohepatic system.

Although the results from the initial cell studies (8, 22, 23) were consistent with respect to the regulation of *Cyp7a1* by LRH-1, they were in apparent contrast with those of subsequent *in vivo* studies using conditional *Lrh-1* deletion (30, 31). Two independent studies showed that *Cyp7a1* messenger RNA (mRNA) levels and protein activity were not reduced upon hepatocyte-specific *Lrh-1* knockout, whereas, as expected, *Cyp8b1* levels were (30, 31). These studies hence suggest that LRH-1 regulates composition and thus physicochemical properties of the bile salt pool but does not control bile salt synthesis rate in mice. Furthermore, heterozygous *Lrh-1* knockout mice exhibited 5-7-fold higher *Cyp7a1* expression levels and increased total bile acid pool sizes (32). Therefore, the proposed role of LRH-1 in the FXR-SHP-LRH-1 cascade, regulating *Cyp7a1* expression, remained uncertain.

It has been speculated that the reason for the discrepancy between *in vitro* and *in vivo* approaches could be a redundant factor that maintains *Cyp7a1* transcription in mice in the absence of LRH-1 (31). As previous *in vivo* experiments were all performed under normal physiological feeding conditions, it is at this stage unclear whether LRH-1 functions as an important transcriptional regulator for *Cyp7a1* expression under conditions in which bile salt synthesis rates must be enhanced to maintain homeostasis, such as during increased fecal bile salt loss.

In this study we describe a novel conditional systemic LRH-1 knockdown mouse model (LRH-1-KD) to evaluate the dependency of bile salt synthesis on LRH-1 under normal, chow-fed conditions, and under conditions of high fecal bile salt loss. Our data show that under physiological (low flux) conditions, LRH-1 determines pool composition rather than bile salt synthesis rate: bile salt synthesis is even slightly increased rather than decreased in LRH-1-KD mice likely due to suppressed ileal *Fgf15* expression. However, using bile salt sequestrants to deplete the bile salt pool by enhancing their fecal excretion, we found that LRH-1 does function as a critical factor in the compensatory induction of hepatic *Cyp7a1* expression and bile salt synthesis. Our data provide mechanistic insight in a missing link in the maintenance of bile salt homeostasis and support the view that LRH-1 functions in a compensatory safeguard mechanism for adequate induction of bile salt synthesis under conditions of high bile salt loss.

MATERIALS AND METHODS

Standard methods and assays can be found in the Supporting Information.

Animals

LRH-1-KD mice were obtained from Taconic Artemis (Cologne, Germany). Details can be found in the Supporting Experimental Procedures. Twenty to 27-week-old male (n = 8) and female (n = 4) LRH-1-KD mice on the C57BL/6J background and wildtype (WT) male (n = 5) and female (n = 3) littermates were housed in individual cages in a temperature- and light-controlled facility with 12 hours light-dark cycling. All mice were fed commercially available laboratory chow (RMH-B; Hope Farms, Woerden, The Netherlands) containing 200 mg/kg doxycycline (Sigma, St. Louis, MO) and supplemented with colese velam HCl 2% (w/w) (Daiichi Sankyo, Parsippany, NJ) when indicated. All

experiments were approved by the Ethical Committee for Animal Experiments of the University of Groningen. All animals received humane care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health.

Genotyping

Detailed information for genotyping can be found in the Supporting Experimental Procedures.

Experimental animal procedures

All mice were fed chow with 200 mg/kg doxycycline for 4 weeks. Thereafter, mice were transferred for 14 days to chow with doxycycline only or to chow with doxycycline supplemented with 2% (w/w) colesevelam HCl. Weight gain was followed during the course of the study. Mice were anesthetized by intraperitoneal injection of Hypnorm (1 mL/kg) (fentanylcitrate 0.315 mg/mL and fluanisone 10 mg/mL, VetaPharma, Leeds, UK) and diazepam (10 mg/kg) (Centrafarm, Etten-Leur, The Netherlands) and subjected to gallbladder cannulation for 20 minutes as described (35). During bile collection, body temperature was stabilized using an incubator. Bile was stored at −20°C until analyzed. Directly following bile collection, heart puncture was performed under isoflurane anesthesia and animals were sacrificed by cervical dislocation.

Blood obtained by heart puncture was collected in ethylenediaminetetraacetic acid (EDTA)-containing tubes. Plasma was stored at −20°C until analyzed. The liver was removed, weighed, and snap-frozen in liquid nitrogen. The intestine was excised, flushed with phosphate-buffered saline, and placed in a Z-form. Three samples of ≈1 cm were removed from the proximal, medial, and distal part of the intestine, representing duodenum, jejunum, and ileum, and snap-frozen in liquid nitrogen. Liver and intestinal samples were stored at −80°C until RNA isolation or biochemical analysis. Fecal excrement was collected from individually housed mice over a continuous 48-hour period. After air-drying, feces were kept at room temperature until analysis.

Analysis in plasma and liver

Triglycerides, cholesterol, free fatty acids were determined with commercial kits. Activities of alanine and aspartate aminotransferases were measured using commer-

cial kits. Quantification of bile salt and neutral sterol species was performed by gas chromatography. Details on analytical measurements can be found in the Supporting Experimental Procedures.

RNA isolation and polymerase chain reaction (PCR) procedures

Gene expression was measured using quantitative PCR (qPCR) performed with a 7900HT FAST system using FAST PCR master mix, Taqman probes, and MicroAmp FAST optical 96-well reaction plates (Applied Biosystems Europe, Nieuwekerk ad IJssel, The Netherlands). Primer and probe sequences can be obtained at RTprimerDB (http://www.rtprimerdb.org) (see Supporting Experimental Procedures for details).

Statistics

All values are presented as Tukey's Box-and-Whiskers plot using median with 25th to 75th percentile intervals (P_{25}-P_{75}). Plots were created using the GraphPad Prism 5 software package. Statistical analyses were performed using SPSS 16.0 (Chicago, IL). Differences between the groups were analyzed by the nonparametric Mann-Whitney *U* test. When multiple comparisons were made (wildtype versus knockdown and chow versus colesevelam), the Kruskal-Wallis *H* test was performed, which was followed by the Conover Posthoc Test using Brightstat.36 Differences were considered statistically significant when $P < 0.05$.

RESULTS

Metabolic parameters in chow-fed conditional systemic LRH-1 knockdown mice

A conditional short hairpin RNA (shRNA) knockdown strategy was utilized to obtain an inducible and reversible whole body *Lrh-1* knockdown model. The model is based on a shRNA sequence targeting *Lrh-1* (NR5A2) cloned behind a doxycycline-responsive promoter. The construct is targeted at the Rosa26 locus along with the enhanced tet-repressor (Figure 1A). The resulting C57BL/6J mice were bred to be heterozygous for the knockdown cassette and WT littermates lacking the targeting construct were used as controls. *Lrh-1* gene knockdown was induced by doxycycline administration by way of the food for 5 weeks. As shown in Figure 1B, hepatic *Lrh-1* mRNA levels were reduced by ≈90%-95%, whereas the reduction of *Lrh-1* expression in small intestine was ≈60%-

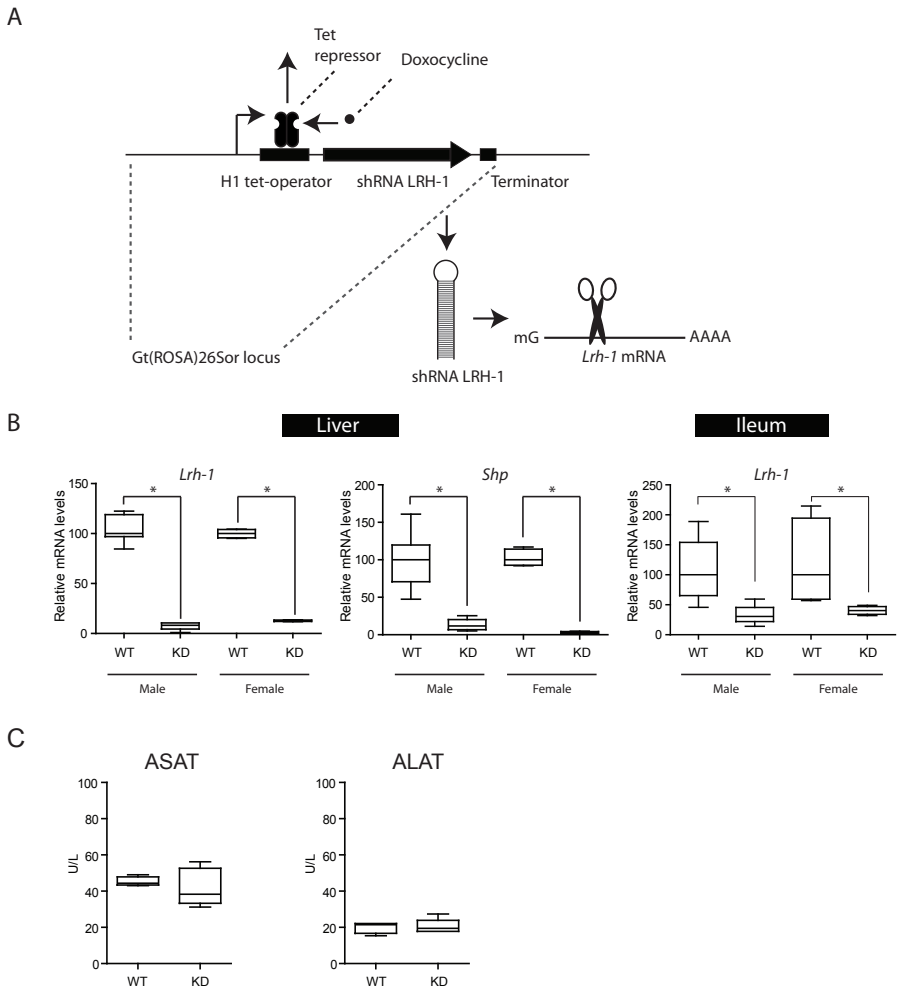

Figure 1. Efficient knockdown of LRH-1 in a novel LRH-1 transgenic mouse model. (A) Schematic representation of the Tet-inducible LRH-1-KD system. (B) Gene expression shows a strong reduction in mRNA levels of *Lrh-1* and the LRH-1 target-gene *Shp*. (C) Plasma aspartate aminotransferase (ASAT) and alanine aminotransferase (ALAT) levels were not altered in LRH-1-KD mice compared to WT littermates (n = 3-8 animals per group) (*$P < 0.05$).

70% in male and female mice (Figure 1B). The expression of *Shp*, a well-established *Lrh-1* target gene (22, 23) was robustly reduced in liver (Figure 1B). In contrast, levels of steroidogenic factor-1, the closest paralog of LRH-1, in the ovaries were unaltered upon expression of the shRNA (data not shown), indicating that knockdown is specific for *Lrh-1*. There were no overt abnormalities noticed in either group. Plasma aspartate

aminotransferase and alanine aminotransferase activities were unchanged (Figure 1C), implying that knockdown of hepatic *Lrh-1* has no detrimental effect on hepatocyte cell integrity. As our model is fundamentally different from two previously reported ones (30, 31), we first analyzed a number of general metabolic parameters. As shown in Supporting Table 1, plasma cholesterol and triglyceride levels were unaltered and plasma lipoprotein profiles were found to be unchanged between wildtype and knockdown animals (data not shown).

LRH-1 knockdown affects bile salt composition and bile salt synthesis under chow-fed conditions

Two previous reports showed that bile salt composition rather than synthesis rate was altered in liver-specific *Lrh-1* knockout mice (30, 31). Consistent with this, hepatic *Cyp7a1* mRNA levels remained unaltered or were even slightly induced, whereas those of *Cyp8b1* were reduced. We also found that knockdown of LRH-1 resulted in a significant reduction of *Cyp8b1* mRNA levels (Figure 2A). Surprisingly, hepatic *Cyp7a1* mRNA levels were increased upon LRH-1 knockdown (Figure 2A). Several genes implicated in hepatic bile salt transport (*e.g.*, *Ntcp, Abcb11/Bsep*, and *Abcb4/Mdr2*) were all mildly reduced upon LRH-1 knockdown (Figure 2A), in agreement with previous findings (31).

We next tested whether the physicochemical properties of the neutral sterol fraction as well as the bile salt pool were affected upon LRH-1 knockdown. LRH-1 knockdown did not significantly alter amounts or relative abundances of each of the major neutral sterols in feces (Supporting figure 1A-C). In agreement with induced *Cyp7a1* levels, the total amount of fecal bile salts secreted per day, reflecting hepatic synthesis, was slightly increased (males +57%, females +59%) (Figure 2B). The primary bile salts cholate (CA) and chenodeoxycholate (CDCA) are the direct products of *de novo* bile salt synthesis. Modifications of these bile salts in liver and intestine give rise to differentially structured primary and secondary bile salts, respectively. Consistent with suppressed hepatic *Cyp8b1* expression levels, the profile was shifted towards CDCA-derived bile salts relative to CA-derived bile salts (Figure 2C). Specifically, fecal contents of deoxycholate (DCA) were greatly reduced (Figure 2D), whereas the relative and absolute abundances of CDCA and α-muricholate were increased (Figure 2D). These data show that bile salt synthesis is shifted towards the CDCA production upon LRH-1 knockdown, in agreement with previous findings (30, 31). For most of these observations, no gender

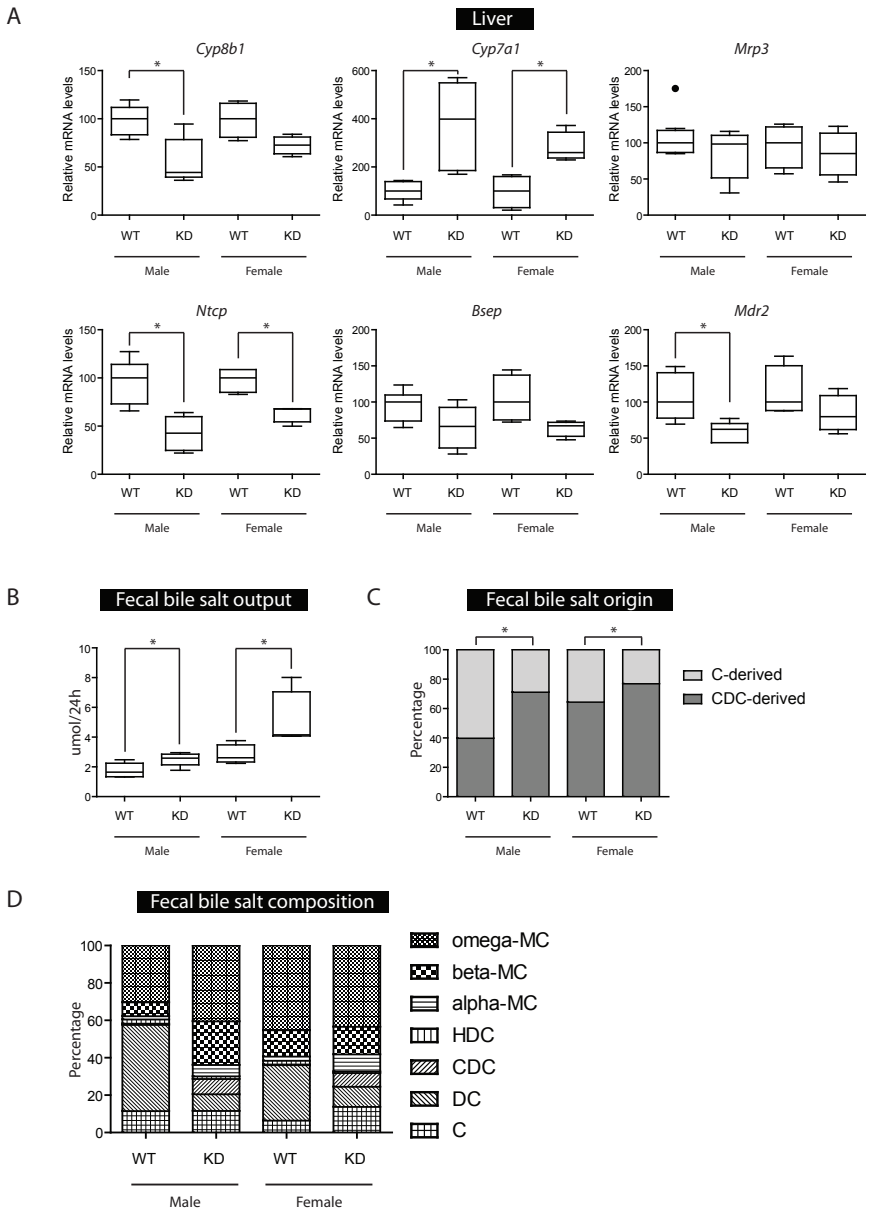

Figure 2. LRH-1-KD in chow-fed animals reduces Cyp8B1 expression and changes the physicochemical properties of the bile salt pool. (A) LRH-1-KD altered gene expression of LRH-1 target genes. (B) LRH-1-KD caused a small but significant increase in fecal bile salt excretion. (C) LRH-1-KD induces a shift in the relative abundance of fecal CA versus CDCA-derived bile salts. (D) Relative abundance of fecal bile salt species in WT versus LRH-1-KD animals (n = 3-8 animals per group) (*$P < 0.05$). CA, cholate; DCA, deoxycholate; CDCA, chenodeoxycholate; HDCA, hyrodeoxycholate; alpha-MCA, alpha-muricholate; beta-MCA, beta-muricholate; omega-MCA, omega-muricholate.

differences were observed. However, fecal bile salt composition was slightly different between males and females under chow-fed conditions (Figure 2D).

LRH-1 is critical for upregulation of *Cyp7a1* expression during bile acid sequestration

As LRH-1 seems to be dispensable for maintenance of *Cyp7a1* expression under chow-fed conditions, we evaluated whether LRH-1 is essential for upregulation of *Cyp7a1* expression under conditions when high rates of bile salt synthesis are required to compensate fecal loss. Colesevelam-HCl is a widely used bile salt sequestrant and its administration massively induces fecal bile salt excretion in mice without affecting pool size (33). LRH-1-KD and WT littermates were fed chow with doxycycline for 4 weeks to induce LRH-1 silencing. Thereafter, mice were fed doxycycline-containing chow with or without colesevelam for 2 weeks. Also in this experiment, *Lrh-1* mRNA levels were robustly reduced in livers of LRH-1-KD animals and reduced to about 60% to 40% along the small intestinal tract (Figure 3A). Colesevelam results in enhanced conversion of hepatic cholesterol to bile salts that must be compensated for by induction of *de novo* cholesterol synthesis by way of upregulation of HMG-CoA reductase (HMGCR), the rate-controlling enzyme of cholesterol synthesis. Indeed, robust *Hmgcr* induction was observed in the colesevelam-treated WT mice (Figure 3B). Colesevelam treatment did not alter hepatic *Lrh-1* expression but reduced hepatic *Shp* levels in wildtypes (Figure 3C). Consistent with a previous report (31) we found a small but significant reduction in hepatic *Fxr* mRNA levels in LRH-1-KD mice (Supporting figure 2A), whereas small intestinal *Fxr* mRNA levels were unaltered (Supporting figure 2B). Colesevelam did not alter hepatic or intestinal *Fxr* expression (Supporting figure 2A, B). Hepatic *Hnf4*α transcript levels were also slightly reduced in LRH-1-KD mice, whereas those of the Liver X receptor (*Lxr*α), a nuclear receptor involved in *Cyp7a1* transcription in mice (34), were found unchanged (Supporting figure 2A).

In agreement with data from the previous experiment, knockdown of LRH-1 resulted in an increase of hepatic *Cyp7a1* expression (Figure 3C). Interestingly, whereas colesevelam treatment resulted in the expected and robust increase of *Cyp7a1* transcription in wildtype mice, such an induction was not observed in the knockdown animals (Figure 3C). Rather, hepatic *Cyp7a1* mRNA levels were comparable in knockdown animals on and off colesevelam. The same pattern was seen for *Hmgcr* expression (Figure 3B). As in the first experiment, *Cyp8b1* mRNA levels were reduced in the LRH-1-KD animals.

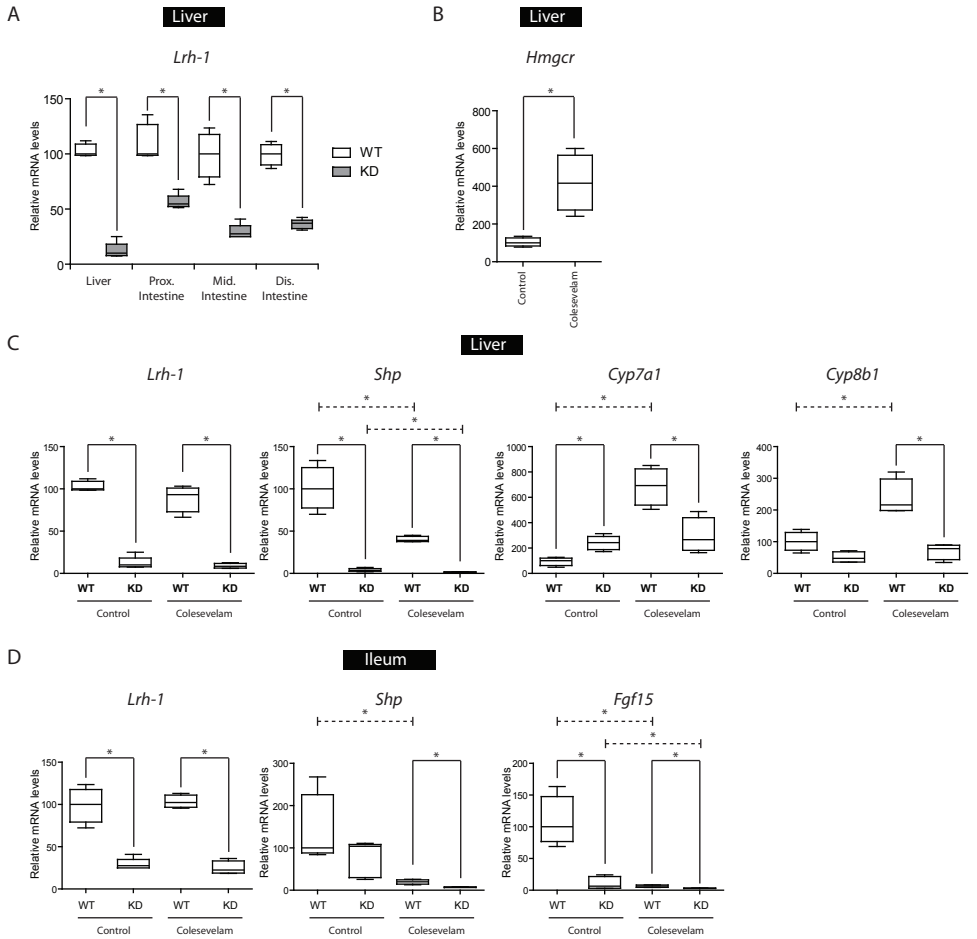

Figure 3. LRH-1-KD animals cannot adequately up-regulate *Cyp7a1* expression. (A) Gene expression shows a strong reduction in mRNA levels of *Lrh-1* in LRH-1-KD animals. (B) Colesevelam-mediated fecal bile salt excretion up-regulates HMG-CoA reductase expression. (C) Hepatic gene expression of WT versus colesevelam-treated animals. Colesevelam induces *Cyp7a1* and *Cyp8b1* in WT animals but not in LRH-1-KD animals (D) Ileal gene expression of WT versus colesevelam-treated animals. LRH-1-KD causes a reduction of ileal *Fgf15* expression (n = 4-5 animals per group) (*$P < 0.05$).

Transcription of the *Cyp8b1* gene was tremendously induced upon colesevelam treatment in the wildtype but not in the knockdown animals (Figure 3C).

These results show that LRH-1 is a critical transcription factor for adequate upregulation of *Cyp7a1* and *Cyp8b1* transcription under conditions of bile salt sequestration. In addition, the apparent paradoxical behavior observed for *Cyp7a1* transcription in

the LRH-1-KD mice suggest that two LRH-1-dependent, but mechanistically different, mechanisms are involved in the transcriptional regulation of *Cyp7a1* expression.

A previous study in mice deficient for intestinal *Lrh-1* showed a reduction of intestinal *Fgf15* mRNA expression, suggesting that intestinal LRH-1 directly or indirectly regulates *Fgf15* expression (31). Colesevelam did not alter intestinal *Lrh-1* expression in wildtype mice but did suppress *Shp* and *Fgf15* expression (Figure 3D), which is consistent with previous findings (33). Intestinal *Shp* levels were significantly reduced in LRH-1-KD mice on and off colesevelam (Figure 3D). Interestingly, we also found a tremendous reduction in *Fgf15* mRNA levels in Lrh-1-KD mice on and off colesevelam, indicating that (intestinal) Lrh-1 regulates the expression of the *Fgf15* gene. To further support this relationship, we tested whether LRH-1 would increase expression of *FGF19*, the human ortholog of murine FGF15, in DLD cells. Transduction of DLD cells with increasing amounts of recombinant LRH-1 encoding adenoviral particles (Supporting figure 3A,B) caused a dose-dependent increase in *FGF19* mRNA expression (Supporting figure 3C). These data indicate that LRH-1 indeed induces *Fgf15/19* expression.

Alterations in bile salt metabolism in LRH-1-KD mice during bile acid sequestration

We tested whether altered *Cyp7a1* expression in colesevelam-treated LRH-1-KD animals also had physiological consequences. Knockdown of LRH-1 did not cause significant alterations in bile flow rate and only tended to reduce biliary bile salt output (Figure 4A, B). Treatment with colesevelam did not affect bile flow, but reduced biliary bile salt output in both WT mice and LRH-1-KD mice (Figure 4A, B), in agreement with previous studies from our laboratory (33). In agreement with the observed increase in *Cyp7a1* expression levels (Figure 3C), knockdown of LRH-1 caused a modest increase (+10%) of fecal bile salt output (Figure 4C). As expected, sequestrant treatment led to a massive induction (+272%) of fecal bile salt output in WT mice. Because colesevelam was given for 2 weeks, a new steady state is established in which fecal loss depicts enhanced bile acid synthesis. In LRH-1-KD mice there was no increase in fecal bile acid output after 2 weeks (Figure 4C), indicating that LRH-1-KD mice cannot upregulate bile acid synthesis during colesevelam treatment.

As *Cyp8b1* expression was also dysregulated in LRH-1-KD mice, we expected that LRH-1 knockdown combined with sequestrant would have profound effects on bile salt composition. Supporting figure 4 provides details on both the relative and abso-

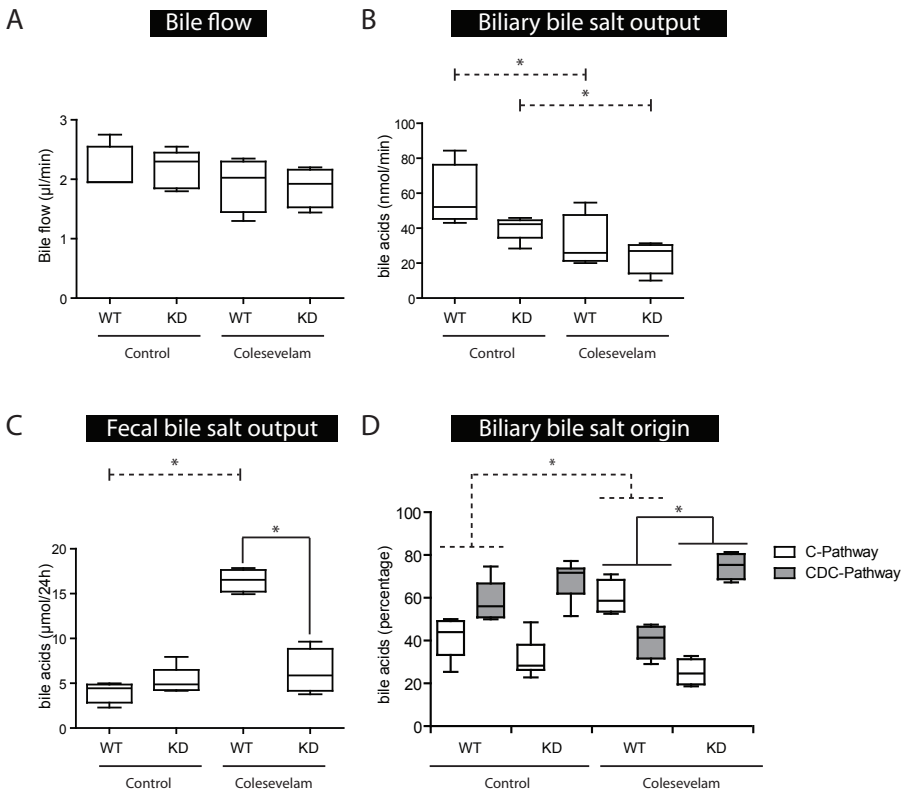

Figure 4. LRH-1-KD animals cannot adequately up-regulate bile salt synthesis. (A) Bile flow was unchanged whereas (B) biliary bile salt output was slightly reduced in colesevelam-treated animals. (C) Fecal bile salt excretion is strongly induced in WT animals but not in LRH-1-KD mice reflecting a lack in the up-regulation of bile salt synthesis in LRH-1-KD mice. (D) Synthesis of CA-derived bile salts was massively increased upon colesevelam treatment in WT but not LRH-1-KD mice (n = 4-5 animals per group) (*$P < 0.05$).

lute fecal and biliary bile salt compositions. In agreement with previous findings (33) colesevelam treatment resulted in increased relative and absolute contents of fecal DCA (Supporting figure 4A, B). Under sequestrant-fed conditions, the loss of bile salts is mainly compensated by an increased hepatic synthesis of CA that results in an increased relative abundance of CA-derived bile salts in bile (Figure 4D and Supporting figure 4C, D). However, LRH-1-KD animals cannot compensate for the sequestrant-induced loss of bile salts by upregulating CA and DCA synthesis (Supporting figure 4B) and this results in a decrease in the relative abundance of CA-derived bile salts and an increase in the relative abundance of CDCA-derived bile salts in bile (Figure 4D, Supporting figure 4C, D).

DISCUSSION

LRH-1 is a nuclear receptor that regulates the expression of a variety of genes involved in cholesterol and bile salt metabolism. Cultured cell studies have shown that both CYP7A1 and CYP8B1, two key enzymes in bile salt synthesis, are regulated by LRH-1. *Cyp7a1* was initially identified as an LRH-1 target gene in an unbiased screen (8). Subsequent cell studies showed that LRH-1 acts as a positive transcription factor as well as a docking site for the transcriptional repressor SHP (22, 23). Comprehensive analysis of the physiological importance of LRH-1 *in vivo* has been hampered by the embryonic lethality of *Lrh-1* knockout mice. Two laboratories independently generated conditional liver-specific *Lrh-1* knockout models (30, 31). Surprisingly, hepatocyte-specific deficiency of *Lrh-1* had no significant effect on *Cyp7a1* expression (30, 31), and heterozygous *Lrh-1* knockout mice exhibited 5 to 7-fold higher Cyp7a1 expression levels (32). Proposed explanations for these surprising findings were that LRH-1 either does not regulate *Cyp7a1 in vivo*, or that compensatory responses or redundant factors maintain *Cyp7a1* expression in the absence of LRH-1 (31). In this study we used conditional whole-body LRH-1 knockdown mice to establish the involvement of LRH-1 on *Cyp7a1* transcription *in vivo*. Our data unequivocally demonstrate that LRH-1 is a critical transcription factor that is required for adequate upregulation of *Cyp7a1* expression under conditions associated with high fecal bile salt loss, as caused by sequestrant treatment. Hence, the inability to upregulate *Cyp7a1* expression translated into relatively low bile salt synthesis rates in LRH-1 knockdown animals compared to wildtypes during sequestrant treatment. Together, our data resolve the apparent discrepancy between the outcomes of *in vitro* cell studies (8, 22, 23) and *in vivo* mouse studies (30, 31). This proves the previously predicted role of LRH-1 in CYP7A1 expression and complements the proposed mechanism of bile acid inhibition of CYP7A1 expression by way of the FXR-SHP-LRH-1 cascade. In this pathway bile acid activation of FXR leads to induction of SHP, which in turn inhibits CYP7A1 activation by LRH-1. In agreement with cell studies (26) and previous *in vivo* studies (30, 31), our data demonstrate that LRH-1 is critical for maintenance of *Cyp8b1* expression, also under normal feeding conditions. Our data also show that LRH-1 is critical for adaptation of *Cyp8b1* expression during high bile salt loss. In physiological terms, the reduction of *Cyp8b1* expression levels in the knockdown animals

was accompanied by the anticipated proportions of CA-derived versus CDCA-derived bile salts in bile and feces.

Together, the data clearly indicate that *Cyp7a1* and *Cyp8b1* expression are differentially regulated. LRH-1 appears to be critical for both *Cyp7a1* and *Cyp8b1* transcription under conditions of high bile salt loss yet dispensable for *Cyp7a1* but not for *Cyp8b1* expression under "normal" conditions. This strongly indicates that compensatory mechanisms or redundant transcription factors exist for maintenance of *Cyp7a1* expression. Indeed, we and others showed that several transcription factors, including LXR/RXR, HNF4alpha and SHP contribute to *Cyp7a1* transcription (Supporting figure 5). Unfortunately, several attempts to study *Cyp7A1* and *Cyp8B1* promoter occupancy by LRH-1 and HNF4alpha using chromatine immunoprecipitation analysis on liver material failed. Therefore, the nature of the differential regulation for *Cyp7a1* and *Cyp8b1* under normal conditions remains obscure and can even be mediated by epigenetic regulators such as GPS2 (37).

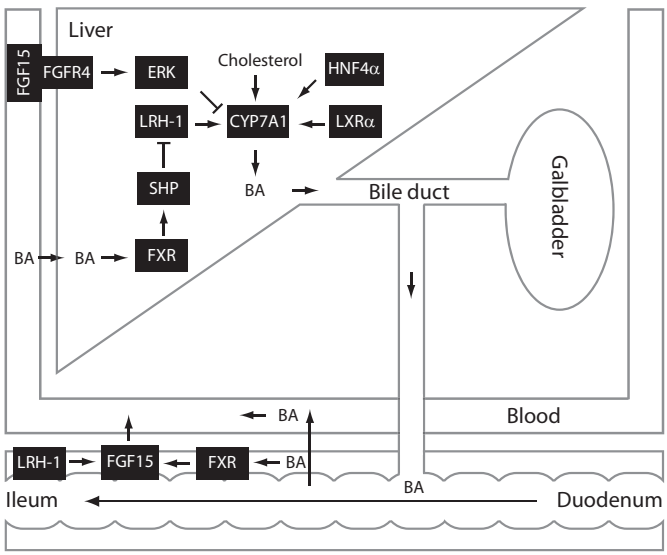

Figure 5. Lrh-1 regulates *Cyp7a1* expression from two distinct sites in the enterohepatic circulation. Schematic representation of physiological pathways that regulate *Cyp7a1* transcription. Hepatic LRH-1 positively contributes to *Cyp7a1* transcription, whereas intestinal LRH-1 represses *Cyp7a1* transcription by way of the induction of *Fgf15* expression.

Careful examination of our data revealed that systemic knockdown of LRH-1 actually resulted in a significant upregulation of hepatic *Cyp7a1* expression that was accompanied by a small increase of bile salt synthesis. This indicates that two different pathways with a reciprocal outcome modulate *Cyp7a1* expression in our model. *Lrh-1* was significantly reduced in the small intestine of LRH-1-KD mice and, in agreement with the results from a conditional intestinal *Lrh-1* knockout model (31), we also found that intestinal *Fgf15* expression was significantly reduced. Experiments in DLD cells further support evidence that LRH-1 modulates *FGF19* expression. However, it remains to be elucidated whether these effects result from a direct transcriptional induction by LRH-1, or by way of indirect mechanisms.

Surprisingly, Lee et al (31) reported that the reduction of intestinal *Fgf15* expression in intestine-selective *Lrh-1* knockouts did not result in an altered hepatic *Cyp7A1* expression. However, the reduction of intestinal *Fgf15* expression was relatively mild in these mice and these authors also found that hepatic *Lrh-1* knockout resulted in a reduction of intestinal *Fgf15* expression, possibly as a result of a reduction in FXR agonist activity in the hepatic *Lrh-1* knockout mice (31). Thus, the separate deletion of either hepatic or intestinal *Lrh-1*, each reducing intestinal *Fgf15* expression levels, appears not to alter hepatic *Cyp7a1* expression levels. Yet when combined, as is the case in our LRH-1-KD mice, the reduction of *Fgf15* expression is strong enough to affect hepatic *Cyp7a1* expression. Indeed, *Lrh-1* haploinsufficiency resulting in a whole-body reduction of LRH-1 showed higher *Cyp7a1* levels compared to littermates harboring both alleles (9). This provides additional insights into the central role of FGF15 in bile acid homeostasis. Interestingly, our data show that only *Cyp7a1* and not *Cyp8b1* is induced upon LRH-1 knockdown. The involvement of *Fgf15* herein is supported by data from Kim et al. (38), who showed that *Cyp7a1* is suppressed much more efficiently compared to *Cyp8b1* by FGF15 signaling.

In summary, our data demonstrate that LRH-1 is a critical transcription factor for upregulation of *Cyp7a1* expression and bile salt synthesis *in vivo* during bile salt sequestration. In addition, our data support the view that LRH-1 affects *Cyp7a1* expression from at least two sites in the enterohepatic system. Hepatic LRH-1 together with other transcription factors positively regulates *Cyp7a1* expression, whereas intestinal LRH-1 causes an opposing effect by stimulating the expression of *Fgf15* expression in enterocytes resulting in a repression of CYP7A1 (Figure 5). The finding that LRH-1 is

indispensable for upregulating bile salt synthesis indicates that it could serve as an attractive target to combat hypercholesterolemia.

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